FINAL REPORT

for

Determination and Microbial Survival of Cryogenic Whole Air Sampling Conditions

27 December 1964—27 July 1965

Contract No.: NAS5-9529

Prepared by

MELPAR, INC.
7700 Arlington Boulevard
Falls Church, Virginia

for

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
GODDARD SPACE FLIGHT CENTER
Greenbelt, Maryland

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1. INTRODUCTION

Under Contract NAS5-9529 for NASA's Goddard Space Flight Center, a series of experiments has been conducted to qualitatively determine the survival of eight species of microorganisms to shock wave exposure and subsequent cryogenic whole air sampling. The results of these experiments are to be applied in the design of systems to be used for sampling the microbial content of the upper atmosphere.

The eight microorganisms chosen for these experiments are Cladosporium resinae, Aspergillus niger, Penicillium notatum, Bacillus globigii, Serratia marcescens, Clostridium pasteurianum, Nocardia asteroides, and Streptomyces griseus. Several basic considerations were made in selecting these microorganisms. The first was to include microorganisms which are known to vary in their ability to survive higher temperatures. In general, spores are more resistant to heat than vegetative forms. Second, microorganisms which vary in their oxygen requirements for metabolism were included in the selection. Aerobes, anaerobes, and microorganisms indifferent to the presence of molecular oxygen were selected. Variations in the size of the microorganisms was a third consideration as the propagation of the particle in a shock wave could vary with the size of the particle. A fourth consideration was to choose microorganisms producing different colored growth, thus facilitating accurate identification. Fifth, all the microorganisms selected were nonpathogens, therefore, eliminating the need for special precautions.

The physical conditions to which these microorganisms were subjected were chosen from those suggested by the design of an existing collector system[1] to be used in sampling the dust content of the upper

atmosphere. This system utilizes a low-temperature ($4^{o}K$-$30^{o}K$) metal surface which at high speeds condenses all the air in its path. From this it was assumed that successful collection of microorganisms in the upper atmosphere would be dependent upon the ability of the microorganism to survive:

(a) near vacuum in the upper atmosphere

(b) rapid heating in passage through the shock wave generated by the sampling vehicle

(c) high speed impaction on the metal surface

(d) rapid cooling to a few degrees K

(e) rewarming to room temperature and subsequent storage for a period of time

2. EXPERIMENTAL PROCEDURES

2.1 Preparation of Microorganisms

2.1.1 Purity. Table 1 lists the species of microorganisms selected for study and the characteristics for each species. All eight species were obtained from American-Type Culture Collection. Upon arrival in our laboratory each culture was checked for purity using the standard agar streak plate and dilution plate method. The growth media used were:

(a) Sabouraud dextrose agar for cultures 99, 100, and 226.

(b) Tryptose glucose yeast extract (TGY) agar for cultures 152, 232, and 178.

(c) Anaerobic agar for culture 215.

The culture media, obtained from Baltimore Biological Laboratory, Inc., were prepared and sterilized according to the directions on the label except in the case of Sabouraud dextrose agar where the agar content was increased from 1.5% to 3.0%. Because of the final acid pH of this medium (5.6), 1.5% agar content was not sufficient to give a desirably firm surface. The streak plates were incubated at 28°C and observed daily for the appearance of discrete colony formation. All cultures were incubated aerobically except C. pasteurianum which was incubated anaerobically in a nitrogen gas environment.

Stock cultures were prepared by transferring individual colonies from the streak plates to screw-cap tube agar slants of the appropriate media. These stock cultures were used for subsequent inoculations.

2.1.2 Lyophilization. Each species of microorganism was tested for its ability to survive the process of lyophilization from a water suspension. Screw-cap tube agar slant cultures were used. For cultures 152,

TABLE 1

Characteristics of Selected Microorganisms

Species	Melpar Culture No.	Approximate Spore Size (microns)	Oxygen Requirement	Color of Growth
Cladosporium resinae	99	(ovoid) 4 by 10	Aerobic	Brown
Aspergillus niger	100	(spherical) 9 - 18	Aerobic	Black
Penicillium notatum	233	(spherical) 6 - 12	Aerobic	Green
Bacillus globigii	152	(ovoid) 0.7 by 1.3	Facultative	Orange-Brown
Serratia marcescens	232	none	Facultative	Red
Clostridium pasteurianum	215	(ovoid) 1.5 by 2.0	Anaerobic	Cream
Nocardia asteroides	226	none	Aerobic	Bright Orange
Streptomyces griseus	178	(ellipsoidal) 0.8 by 1.7	Aerobic	White

232, and 215, 72-hour growth was used and for cultures 99, 100, 233, 226, and 178, 10-day growth was used. Water suspensions of the microorganisms of each species were prepared by washing the agar slant culture with 5 ml of sterile distilled water. One milliliter of the water suspension was used for duplicate prelyophilization plate dilution counts.

One milliliter of the water suspension was introduced into a sterile 10-ml vial for lyophilization. Duplicate vials were shell frozen using an acetone-dry ice slurry. These cultures were lyophilized under 0.03 Torr pressure at -55°C. One day after lyophilization, the vials were opened and 2.0 ml of sterile distilled water was added to each. One milliliter of the suspension from each vial was removed and plate dilution counts were made.

Because the vials of lyophilized microorganisms would be stored at room temperature for a period of time before they would be opened and subjected to shock wave temperature, the ability of the lyophilized microorganisms to survive room temperature storage for 32 days was tested.

2.1.3 Mass Cultivation. After demonstrating that all species survived lyophilization, large batches of each culture were prepared. Sixteen-ounce screw-cap prescription bottles were used to cultivate the organisms. Each bottle when lying on the flat side maintained the appropriate $\frac{1}{2}$-inch depth of agar medium. Ten bottles of each culture were prepared. Stock tube agar slant cultures were used as the inocula. All the cultures were incubated at 28°C. Cultures 152, 232, and 215 were permitted to incubate for 72 hours before harvesting and cultures 99, 100, 233, 226, and 178 for ten days before harvesting. Distilled-water

suspensions of each species were prepared. Care was exercised not to include chunks of growth to insure uniform suspensions of organisms and not to dissolve any of the medium in the suspension because a protein carrier was unwanted.

2.1.4 Sample Preparation. The preparation of vials of lyophilized pure-culture and mixed-culture samples was essentially the same as described in section 2.1.2. A total of 48 vials of mixed cultures and a total of 80 vials of pure cultures (10 vials of each of the eight species) were prepared. Each pure-culture vial contained 1.0 ml of the harvested water suspension and each mixed-culture vial contained 0.1 ml of each of the eight species.

Agar plate dilution counts of each culture suspension were made before and after the lyophilization treatment, as described in section 2.1.2.

2.2 Environmental Tests

Before subjecting the microorganism samples to the actual conditions of the shock tube experiments, it was necessary to pre-test the survival of the microorganisms to the 20°K temperature of the collector and to the subsequent warming of the low-temperature sample. In doing this, the immersion time required for the microorganisms to attain the 20°K temperature and secondly, the time required to warm the microorganism to room temperature were determined.

2.2.1 Immersion Time. To determine the required immersion time, a thermocouple was inserted in a test vial containing the lyophilized microorganisms. This vial was evacuated and sealed (Figure 1) and then

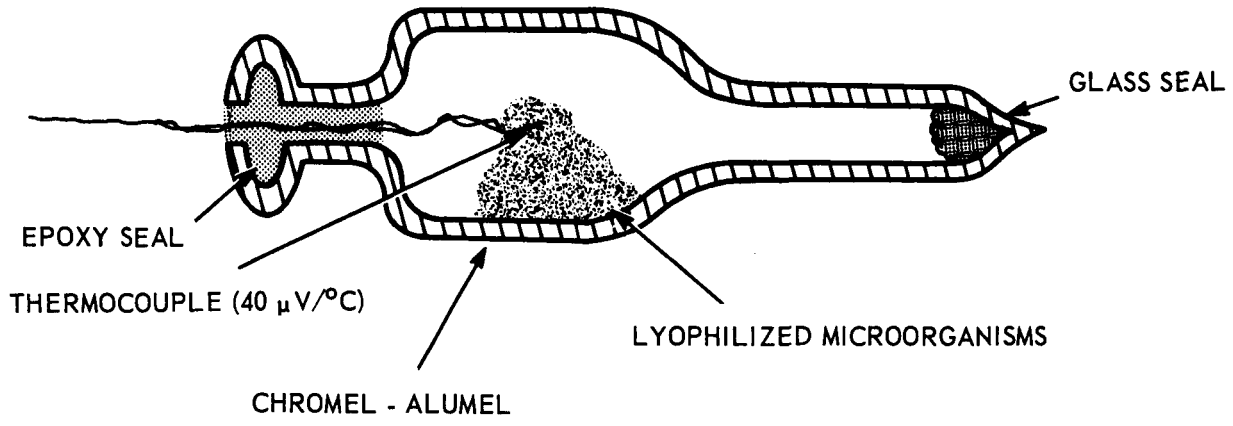

Figure 1. Evacuated Vial Containing Lyophilized Microorganisms and Thermocouple

immersed in liquid nitrogen. Liquid nitrogen was used rather than liquid hydrogen because of the hazard of a hydrogen-air mixture. Taking the average of three trials, a time and temperature relationship curve was determined. The time and temperature relationship curve for liquid-hydrogen immersion was approximated by extending the observed exponential liquid-nitrogen curve to the boiling point of liquid hydrogen.

2.2.2 Warming Time. The length of time required for rapid warming and for slow warming to room temperature were determined in a similar manner. Rapid warming entailed the immersion of the vial, immediately after removal from the liquid nitrogen, into a gallon of water at 25°C. Slow warming was accomplished by allowing the vial to stand at room temperature for 30 minutes after removal from the liquid nitrogen.

2.2.3 Freeze Tests. Because a hydrogen-air mixture is dangerously explosive, this operation was performed outdoors. A 150-liter (23.5 pounds of liquid hydrogen) AIRCO ARHC liquid-hydrogen container was used as the source of supply. A 4½-foot flexible vacuum-insulated hose was used to transfer the liquid hydrogen to a 2 liter open-top Dewar. The entire system (Figure 2) was earth grounded to prevent the possibility of static energy igniting a H_2-air mixture. The withdrawal hose and dip tube were flushed with helium and a positive pressure was maintained before attachment to the withdrawal fitting. The Dewar was also flushed with helium. Liquid hydrogen was then permitted to flow into the Dewar so that it was maintained at full capacity. A total of four pure-culture vials of each of the eight species were immersed in the liquid hydrogen for 12 to 15 minutes. Two of the pure-culture vials of each species were warmed rapidly and two were warmed slowly.

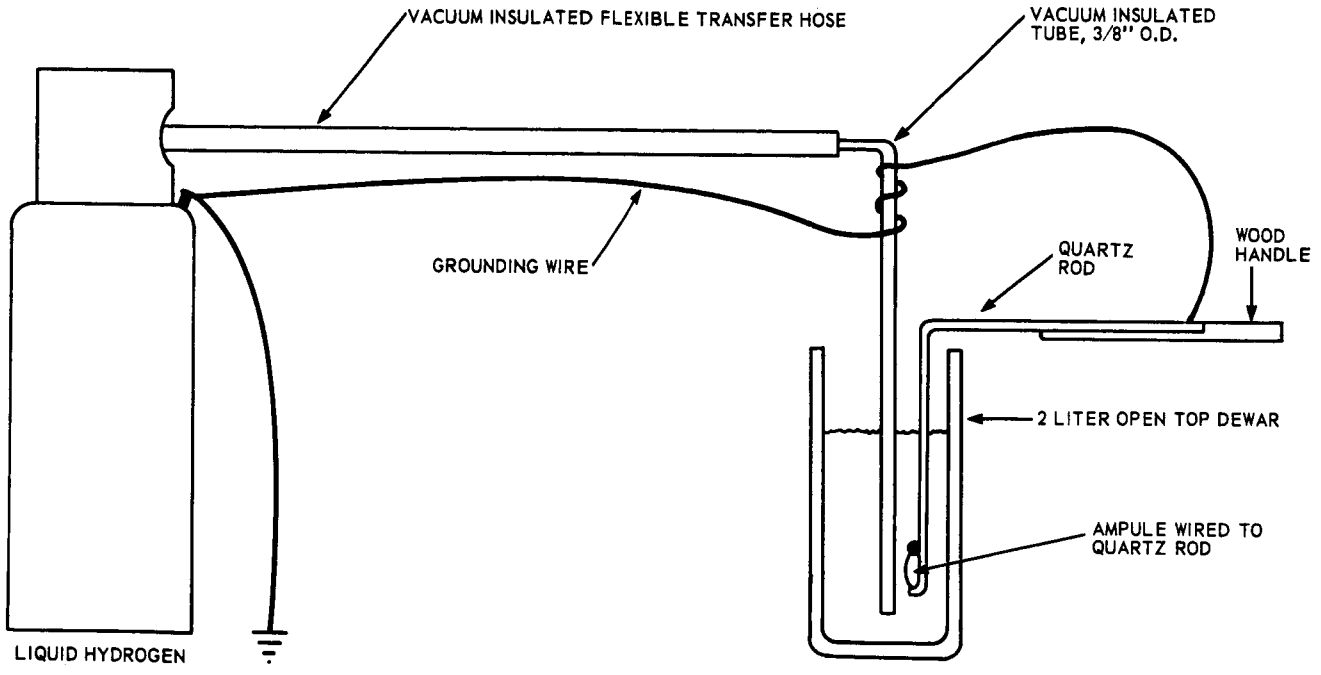

Figure 2. Immersion of Vials in Liquid Hydrogen

2.3 Shock Tube Experiments

2.3.1 Shock Tube Construction. A photograph of the shock tube facility constructed for this program is shown in Figure 3. The shock wave tube was made from 2.5-inch I.D. aluminum tubing with 1/4-inch wall thickness. The driver section was a single tube 5 feet in length. The test section consisted of ten pieces of tubing, each one-foot long to facilitate instrumentation of the tube as well as to permit insertion of the microorganism sample at various distances from the end of the tube. The tube ended in an evacuated dump tank, 12 inches in diameter and 2-feet long, in which the shock wave is dissipated by expansion. The dump tank was connected directly to a 4-inch diffusion pump, so that it was readily pumped below 10^{-3} Torr, the lower limit of the gauge used. Flanges grooved for O rings were welded to the ends of all the pipe sections and the dump tank to permit easy assembly and disassembly. A liquid-hydrogen Dewar mounted in the dump tank held a Millipore filter facing the outlet of the shock tube, so that the organisms carried by the shock wave could impact on the cold filter surface.

The main diaphragm separating the driver gas from the test section was clamped in a ring between the two mating flanges and sealed by another O ring. A shim-brass disk of three-millimeters thickness was found to make a good diaphragm for the 1300 Torr pressure difference.

The disks were scored to a depth such that they would burst spontaneously at a pressure difference of 1600 to 2000 Torr. At 1300 Torr the disks would break and open cleanly when struck by the spring-driven plunger. When the usual four-fold scoring pattern was used to

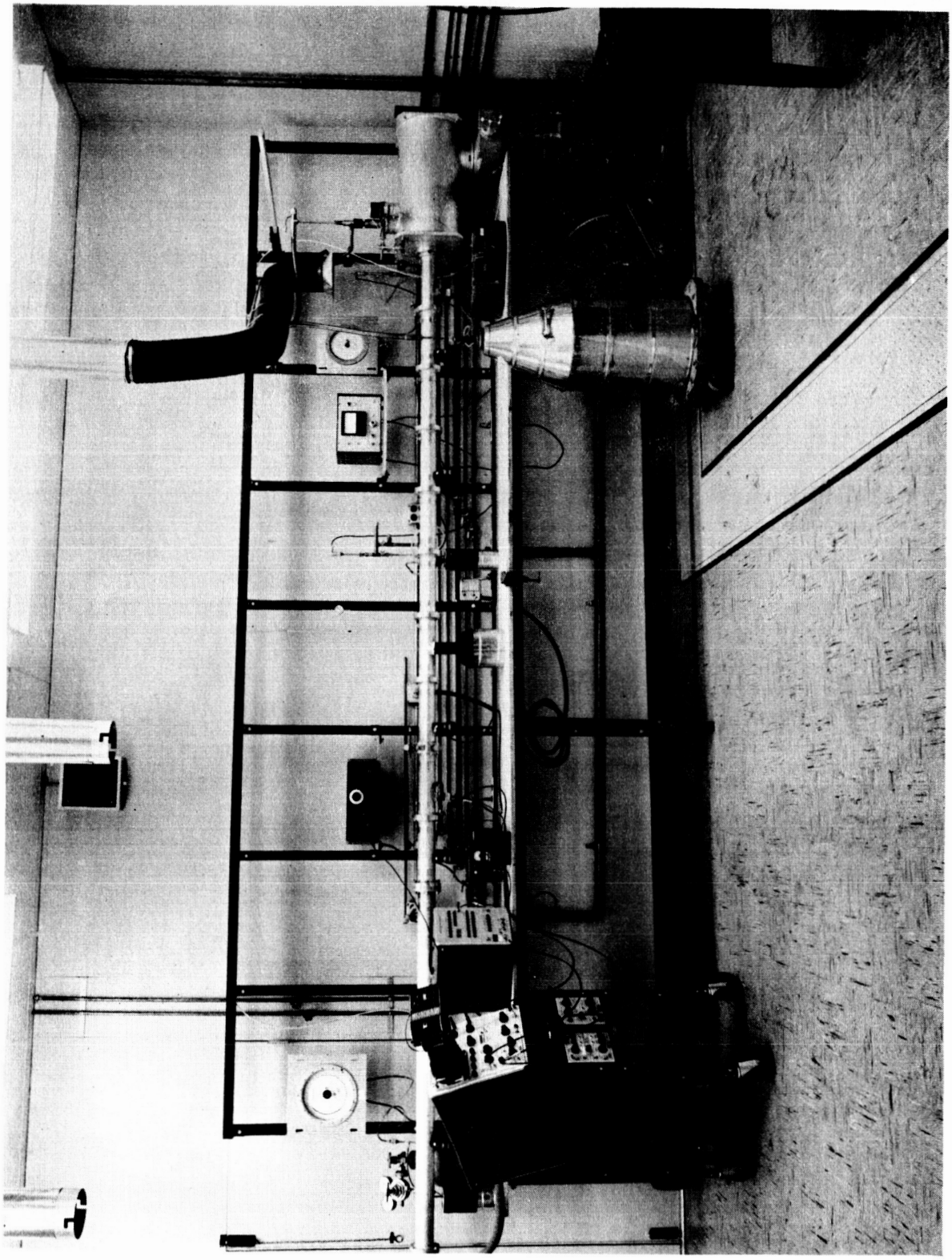

Figure 3. Shock Tube Facility

create a four-petal opening in the disk, it was found that often only two petals opened, giving a considerably reduced shock speed. Use of a three-fold scoring pattern eliminated this problem and gave very good reproducibility in the opening pattern and hence reproducible shock speed for a specified pressure ratio. The scoring cut in the diaphragm brass was made by a spring-loaded glass-cutter wheel adjusted to the proper cutting force. The cutter was simply attached to a wooden block which was run along a guide to produce the three radial score lines in the disk.

A thin collodion or nitrocellulose lacquer membrane, estimated to be from 1-5 microns thick, was used to separate the evacuated dump tank from the test section of the tube containing air at a pressure of 2.5 Torr. A similar but even thinner membrane was used to hold the microorganisms in the path of the shock at the desired location in the test section. Both membranes were mounted on aluminum rings which were sealed with O rings between the tube section flanges. No measurement was made of the amount of shock attenuation upon interaction with these membranes since it has been demonstrated that their effect on the shock strength is negligibly small.[2]

A diagram of the shock tube and pumping system is shown in Figure 4. The sections of the tube separated by the diaphragm and membranes were connected to a common pumping line so that all sections might be evacuated simultaneously to a pressure of about 2×10^{-2} Torr. The pumping connections could then be closed off, and each section filled appropriately. In the present experiments, the driver section was filled with helium dried in a liquid-nitrogen trap, while the test section was

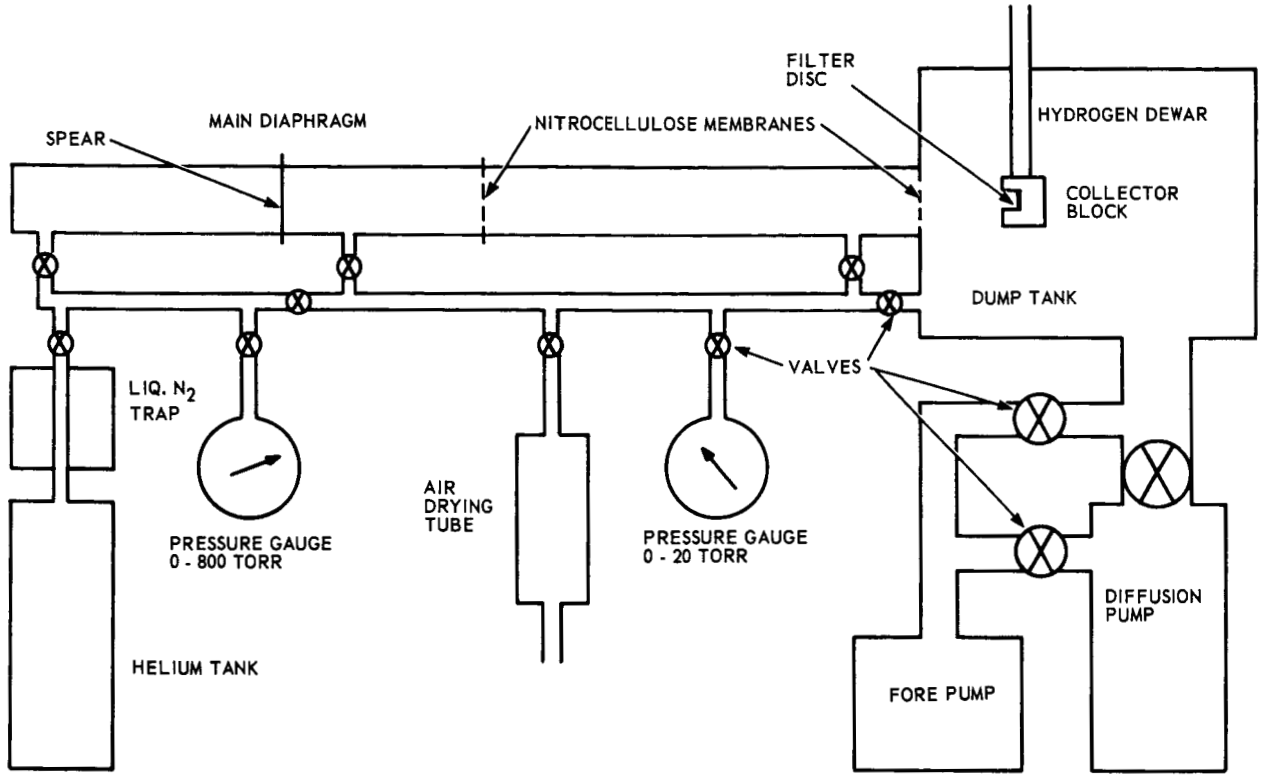

Figure 4. Shock Tube and Pumping Station

filled with room air dried in a liquid-oxygen cooled trap. A Wallace and Tiernan dial manometer of the appropriate pressure range was connected to each of these two sections to provide accurate measurement of the initial pressures.

The shock wave intensity may be roughly predicted from the gas temperature, composition, and pressure ratio across the diaphragm before bursting, but the ultimate check on its intensity is an actual measurement of the shock speed. This was done by a series of ten heat-transfer gauges of conventional design, mounted flush with the inside wall of the tube at one-foot intervals from the diaphragm to the dump tank. Each gauge consisted of a small strip of Mylar tape about 1/2 mm by 5 mm with an evaporated gold coating of about 20- to 50-ohms resistance. The thin gold film acts as a resistance thermometer of extremely short time constant.

A schematic of the shock tube instrumentation of the heat transfer gauges is shown in Figure 5. An oscilloscope driven by a 2-KC function generator provided an unbroken sweep of several milliseconds duration at a rate of 25 μsec/cm, with marker pulses from the time-mark generator every 50 μsec. The oscilloscope trace was triggered by the discharge of a capacitor when the diaphragm breaker needle touched the diaphragm. As the shock wave passed each heat transfer gauge, a step-function signal appeared on the oscilloscope trace, so the time of arrival of the shock could be measured to within a few microseconds. Thus, over a distance of several feet, the shock wave speed could be measured to an accuracy of better than 1%. The reproduction of a typical oscilloscope trace is shown in Figure 6.

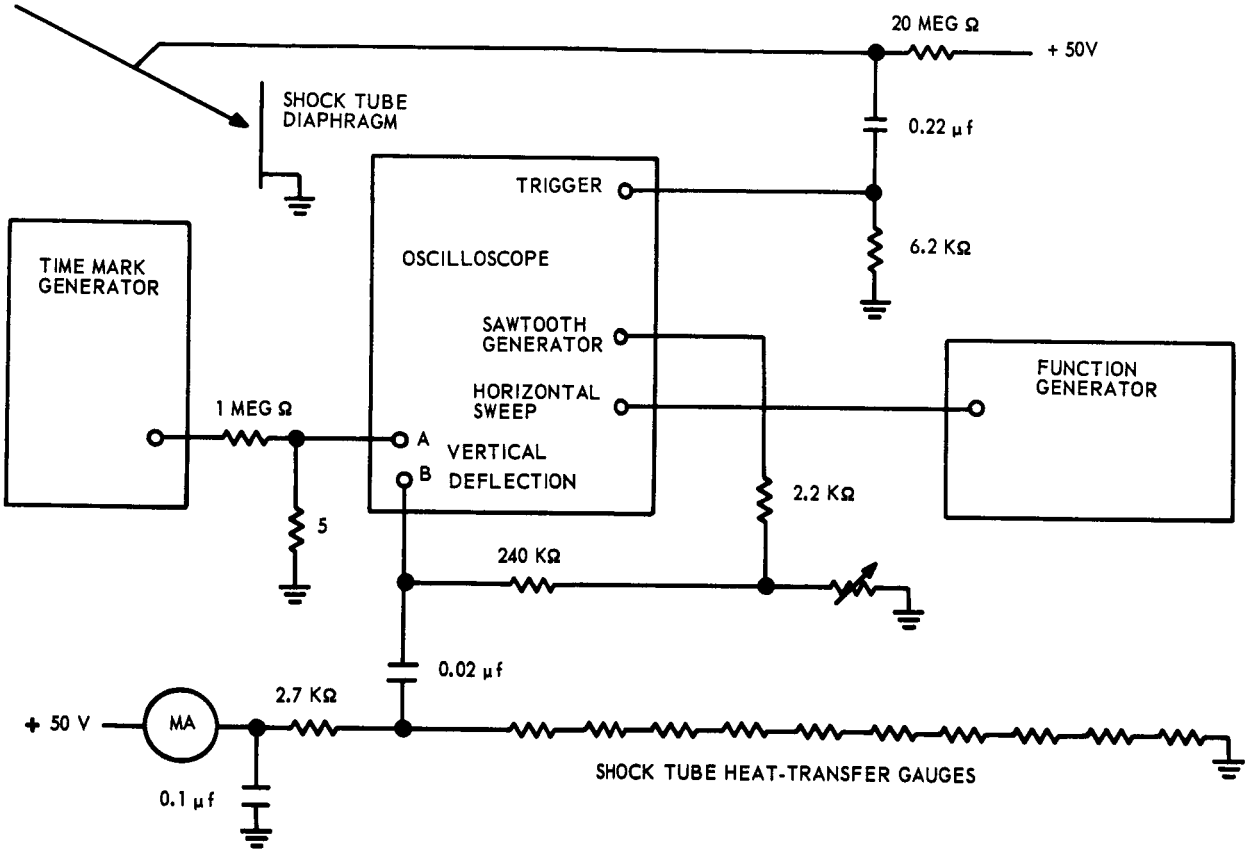

Figure 5. Shock Tube Instrumentation

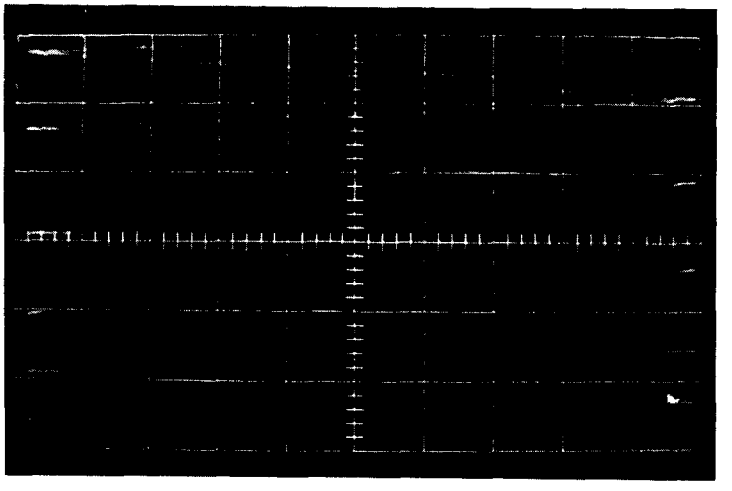

Figure 6. Oscilloscope Record of Shock Velocity

The temperature of the organism collector was monitored with a copper vs. 2.11 atomic percent cobalt-in-gold thermocouple. This thermocouple has a differential output of 16 microvolts per degree at 20°K and was used with a potentiometer sensitive to a few microvolts, so that a temperature drift of one degree could be detected readily. The temperature of the Dewar was taken as 20°K when the thermocouple potential remained steady and the Dewar contained liquid hydrogen.

2.3.2 Shock Tube Calculations. The shocked gas temperature and the contact time of the microorganisms in the heated gas were calculated from the ideal shock wave equations using the measured shock velocity from the heat transfer gauges and the initial temperature, specific heats, and sound velocity of the experimental gas (air).[3] The Mach number of the shock, M_1, is simply the ratio of the shock velocity to the velocity of sound in the gas:

$$M_1 = \frac{V_1}{a}$$

where V_1 = velocity of shock

a = speed of sound

This quantity was determined experimentally from the oscilloscope record of the heat transfer gauges. The calculation of the temperature of the shock-heated air was calculated from the experimentally determined Mach number using the following equation

$$T_2/T_1 = \frac{(\gamma M_1^2 - \frac{\gamma - 1}{2})(\frac{\gamma - 1}{2} \cdot M_1^2 + 1)}{(\frac{\gamma + 1}{2})^2 M_1^2}$$

where T_2 = temperature of shocked gas

T_1 = initial temperature

γ = ratio of specific heats, C_P/C_V

The shock front will travel at a higher speed relative to the tube than the shocked gas and in order to determine the contact time of the samples in the heated gas it is necessary to calculate the gas velocity. The total travel distance divided by the gas velocity will yield the exposure time of the sample. The gas velocity, U, was calculated from the following equation

$$U = \frac{2a}{\gamma + 1}\left(M_1 + \frac{1}{M_1}\right)$$

The temperatures and gas velocities can be reliably calculated from these equations if the final temperature does not exceed approximately 2000°K. This is true for our experiments.

2.3.3 Sample Injection. The shock tube was originally built with a microorganism sample injector mechanism designed to take an extremely fine powdered sample and aerosolize it with a very low pressure force of gas. This sample injector was used in the first test run, but the majority of the sample was too coarse and remained in the injector. The samples upon removal from their evacuated glass storage vials were in the form of loose flakey particles of sizes up to a millimeter or a little more. Attempts to grind the samples only produced much more tightly packed clumps, and it was found that the samples could not be easily sifted.

Another method of sample introduction was adopted for the rest of the runs. The samples were sprinkled directly from the vials onto a thin collodion membrane which was then placed across the tube in the path of the shock. With this method, the individual particles collected were too small to be seen by the unaided eye, so the sample appeared to be adequately aerosolized by the shock.

2.3.4 Shock Tube Sterilization. Sterilization of the entire shock tube would present a considerable problem. However, all that is required is the assurance that the collector filter will not be contaminated by organisms left in the tube from previous runs. The contamination of the tube was checked by blank runs in which no organisms were added to the tube. These blank runs followed immediately after an organism-loaded run with no attempt to clean the tube in the interim. Viability counts on the collection filter showed only a few viable organisms. Hence, no attempt was made to clean the tube between runs, other than by the cleaning action of the shocks themselves. However, one or more blanks were always run before each loaded shock run, and the filter from the last blank was cultured and counted exactly like that of the preceding test organism. The collector assembly that held the filter was autoclaved prior to each microorganism experiment.

2.3.5 Shock Tests. After the first run (Shock run 1) in which the organisms were aerosolized between two collodion membranes immediately before the shock was initiated, the shock tube tests of the microorganisms were conducted as follows. The main diaphragm and the collodion membrane separating the tube from the dump tank were prepared and mounted in the

tube. The Dewar section was autoclaved, a sterile Millipore filter (Millipore Filter Corp., HAWG, 0.45μ, 25 mm) mounted in the channel in the Dewar, and the assembly placed immediately in the dump tank. The vials containing the microorganisms were opened in a dry box and transferred to a collodion membrane which was transported to the shock tube area in a clean culture dish and mounted in the shock tube. The tube was immediately evacuated to approximately 10^{-3} Torr.

The collodion membranes were checked for holes that might have developed during pump down by introducing a small pressure of dry air across them and noting the leak rate. Once it was determined that the membranes did not leak, the Dewar was cooled to 20°K with liquid hydrogen, while continually monitoring the temperature with the copper vs. 2.11 atomic percent cobalt-in-gold thermocouple. When the thermocouple potential stabilized and the Dewar contained liquid hydrogen, the tube was filled to the desired pressure with the dry helium driver gas and the dry air experimental gas. At this point, the shock was initiated by rupturing the main diaphragm by releasing the breaker needle. After the shock, the tube and dump tank were filled with dry air, the Dewar assembly removed, and the Millipore filter placed on the culture medium.

The colonies were counted on sections of plated filter and were multiplied by the appropriate number to estimate the number of colonies on the entire filter. The intent of recording numbers of colonies (Table 5) was not to quantitate the survival of the microorganisms but rather to show the relative numbers of colonies counted.

The shock test procedure was very time consuming, particularly the preparation of the thin membranes, and in order to minimize the number of shock runs, the organisms were first tested under the most severe conditions specified. The initial runs were with mixed samples of microorganisms containing all seven viable organisms. The shock speed was held very close to the 1.56 meters/millisecond required to give an exposure time of 2.0 milliseconds and a temperature of 1200°C. Blank tests were run between each microorganism test to provide a sterilization of the shock tube assembly. A total of 25 runs was performed.

In runs 1 through 6 an initial attempt was made to determine the survival of the mixed organisms to the pre-selected severe conditions. Run 1 of this series was the only test in which the aerosol injector was used, as the injector failed to release all the sample. In plating this series, runs 1 through 4 were plated on TGY agar only, with runs 5 and 6 plated on both TGY and Sabourauds agar. Runs 2, 4, and 5 of this series were blank runs and runs 1, 3, and 6 were mixed-culture tests of cultures 152, 232, 99, 233, 100, 178, and 99.

In runs 8, 10, and 12 the samples were again mixtures of all seven organisms. For these runs and the preceding blanks, the collection filter was broken into sections which were placed separately on three different media: TGY, Sabouraud, and anaerobic agars. In this way, the appropriate medium for the growth of each of the test organisms was provided. All the plates were incubated at room temperature, the TGY and Sabouraud plates aerobically and the anaerobic agar plates anaerobically.

Run 14 was a storage time study of organism 152. The collection filter from this run was broken into four pieces and stored at room temperature for about 1, 10, 100, and 1000 minutes before plating on the TGY agar.

Run 17 was an attempt to run a storage time study on organism 232, but the oscilloscope failed to trigger so that no measure of the shock speed could be obtained.

Run 19 was another test of the survival of organisms 215, 233, and 99. The collection filter from this run was broken into sections which were plated individually on the three agar media.

Run 21 was a test of pure culture 99 with the collection filter sample plated on all three media.

Runs 23 and 25 were attempts to obtain a storage time study on organism 232. In run 25 a portion of the microorganism sample was checked for viability before subjecting it to the shock wave. The collection filter samples obtained from these runs were plated on TGY agar only.

3. RESULTS

3.1 Preparation of Microorganisms

The final selection of the eight species of microorganisms (Table 1) included two substitutions in the preliminary list. Nocardia asteroides (culture No. 226) was substituted for Alternaria brassicae because difficulty was encountered in getting good growth of the Alternaria brassicae, and Streptomyces griseus (culture No. 178) was substituted for Streptomyces thermotolerans because American-Type Culture Collectors had difficulty supplying the desired culture. All the species were tested for purity and pure stock cultures were maintained for subsequent tests.

Preliminary to the preparation of large numbers of mixed-culture and pure-culture sample vials of lyophilized microorganisms, the ability of each species to survive lyophilization from distilled water suspensions was tested. Table 2 shows the results of the preliminary lyophilization experiments. The pre-lyophilization and post-lyophilization counts recorded are averages of duplicate counts. Trial II was performed in the same manner as Trial I. All the cultures satisfactorily survived the lyophilization treatment (between 10 and 95% survival).

Large batches of each of the species were cultivated and water suspensions were made (see section 2.1.3). Microscopic examination of the water suspensions prior to lyophilization showed uniform distribution of the microorganisms and the spore-forming species showed the following spore yields:

TABLE 2

Survival of Eight Species of Microorganisms to Lyophilization from Distilled Water

Species	Culture No.	Pre-lyophilization Count (per ml)	Post-lyophilization Count (per ml)	Percent Survival
Cladosporium resinae	99 Trial I	15×10^5	4×10^5	27
	Trial II	18×10^5	9×10^5	50
Aspergillus niger	100 Trial I	11×10^6	6×10^6	55
	Trial II	10×10^6	3×10^6	34
Penicillium notatum	233 Trial I	2×10^7	0.9×10^7	45
	Trial II	3×10^7	1.2×10^7	40
Bacillus globigii	152 Trial I	5.7×10^9	4.3×10^9	76
	Trial II	7.3×10^9	6.8×10^9	93
Serratia marcescens	232 Trial I	5.5×10^{10}	0.9×10^{10}	16
	Trial II	$4. \times 10^9$	1×10^9	25
Clostridium pasteurianum	215 Trial I	4.2×10^8	4×10^8	95
	Trial II	4.5×10^8	4.1×10^8	91
Nocardia asteroides	226 Trial I	5×10^8	1×10^8	20
	Trial II	2×10^8	0.75×10^8	38
Streptomyces griseus	178 Trial I	8×10^8	3.2×10^8	40
	Trial II	7×10^7	0.7×10^7	10

Cladosporium resinae (No. 99) 88%

Aspergillus niger (No. 100) 96%

Penicillium notatum (No. 233) 94%

Bacillus globigii (No. 152) 65%

Clostridium pasteurianum (No. 215) 50%

Streptomyces griseus (No. 178) 84%

Lyophilized pure-culture sample vials (see section 2.1.4) of each species were tested for viability following room storage. The results of the quantitation of the viability of each species surviving one day and 32 days after lyophilization are shown in Table 3. All lyophilized species showed good survival (31-84%) to room temperature storage except Nocardia asteroides (culture No. 226) which survived one day but not 32 days. Since this microorganism did not survive room temperature storage it was not included in consideration when the mixed-culture vials were tested in the shock wave experiments.

3.2 Environmental Tests

A time-temperature curve was used to determine the immersion time required for the microorganisms to reach 20°K. Since nitrogen was used as the coolant in these experiments, the time-temperature relationship for liquid hydrogen was approximated by extending the curve to the boiling point of liquid hydrogen (see figure 7). From this it was determined that the lyophilized-microorganism vials must be immersed in liquid hydrogen for seven minutes to cool the sample to 20°K.

TABLE 3

Effect of Room Temperature Storage on Lyophilized Pure-Culture Samples

Species	Culture No.	Pre-lyophilization Count (per ml)	1 Day after Lyophilization		32 Days after Lyophilization	
			Count	% Survival	Count	% Survival
Cladosporium resinae	99	10×10^7	6×10^7	60	3×10^7	30
Aspergillus niger	100	7×10^7	1×10^7	14	0.7×10^7	10
Penicillium notatum	233	34×10^6	6×10^6	18	5×10^6	14.7
Bacillus globigii	152	46×10^8	1.5×10^9	33	1×10^9	21.0
Serratia marcescens	232	8×10^9	3.2×10^9	40	1×10^9	12.5
Clostridium pasteurianum	215	7×10^8	2.1×10^8	30	1×10^8	14.3
Nocardia asteroides	226	6×10^6	0.7×10^6	11	0	0
Streptomyces griseus	178	11×10^6	4×10^6	36	3×10^6	27.3

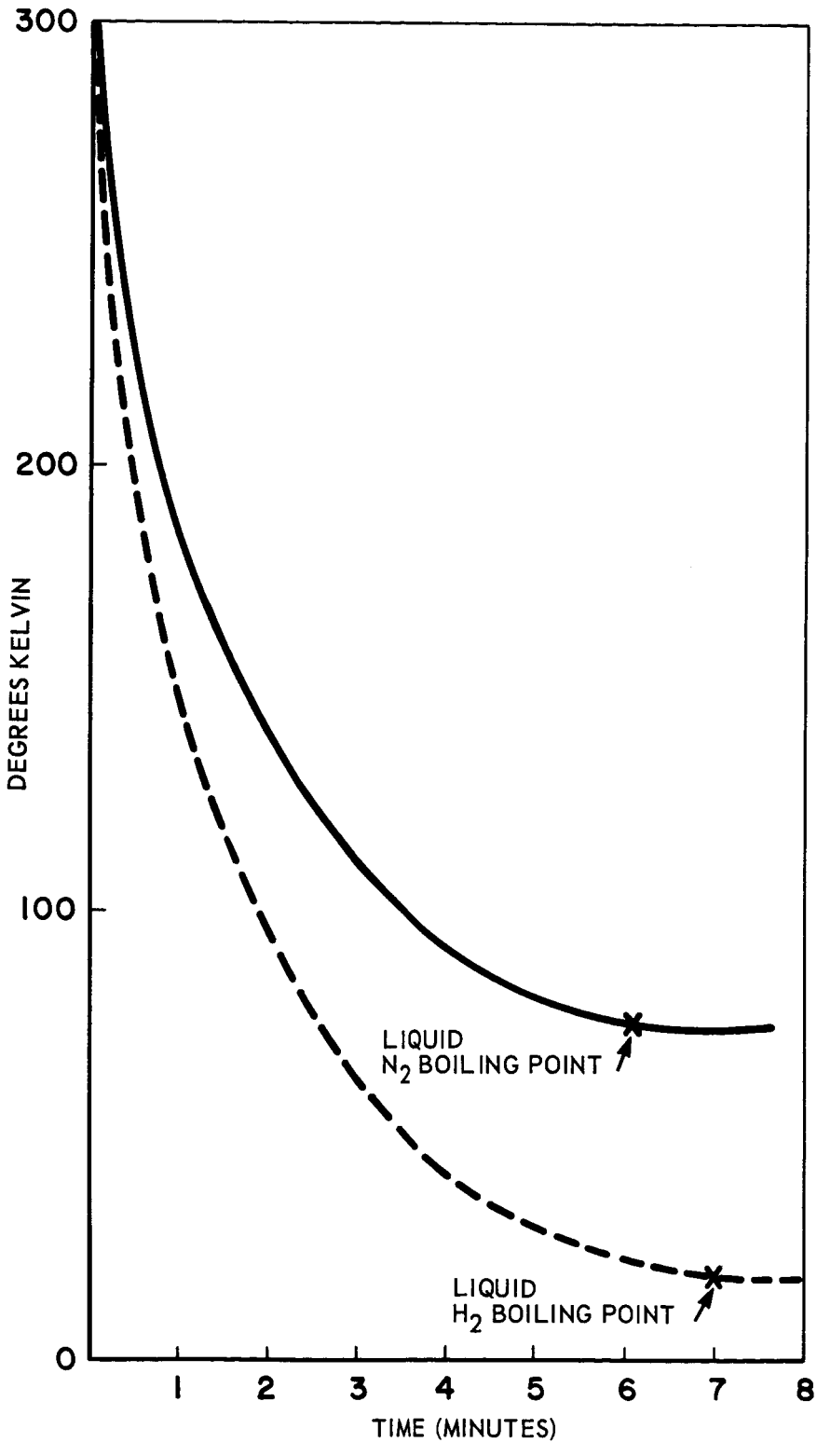

Figure 7. Time and Temperature Relationship After Immersion of Thermocouple Test-Vial in Liquid Nitrogen

The length of time required for the sample to be warmed rapidly or slowly to room temperature was determined in the same manner. From these experiments it was determined that approximately 30 seconds were required for rapid warming and 30 minutes for slow warming.

On the basis of the results obtained from the thermocouple measurements in liquid nitrogen, the pure-culture sample vials were immersed in liquid hydrogen for 12 to 15 minutes, thus maintaining the cells at 20°K for 5 to 8 minutes. Duplicate vials of each species were then both warmed rapidly and slowly to room temperature. The vials were opened 6 to 18 hours later and 2 milliliters of sterile distilled water was added to each vial. One milliliter of the water suspension was withdrawn and plate counts using the appropriate media were made. The average of duplicate counts were recorded. Table 4 shows the results of this experiment. All the test organisms except Culture No. 232 showed good survival (20-100%) after exposure to 20°K and either rapid or slow warming to room temperature. Culture 232 showed 0.2% survival after slow warming and 0.012% survival after rapid warming. Although this was a drastic reduction in survival, a large number of the microorganisms (1.2×10^5) remained viable.

3.3 Shock Tube Experiments

The results of the shock treatment of microorganisms are shown in Table 5. The culture plates were observed periodically for the appearance of colonies. The times of observation varied somewhat depending upon when the organism-impregnated Millipore filter was cultured. However, all were observed daily and usually several times

TABLE 4

Survival of Lyophilized Microorganisms After 5- to 8-Minutes Exposure at 20°K Followed by Rapid and Slow Warming*

Culture No.	Control Count Not Cooled (per vial)	Rapid Warming		Slow Warming	
		Count (per vial)	% Survival	Count (per vial)	% Survival
99	3×10^7	2×10^7	67	6×10^6	20
100	7×10^6	7×10^6	100	8×10^6	114
233	5×10^6	2×10^6	40	1.4×10^6	28
152	1×10^9	9×10^8	90	7×10^8	70
232	1×10^9	1.2×10^5	0.012	2×10^6	0.2
215	1×10^8	4×10^7	40	2×10^7	20
178	3×10^6	4×10^6	100	2.4×10^6	80

*Total exposure in liquid hydrogen was 12-15 minutes. The cells were maintained at 20°K however, for only 5-8 minutes. In rapid warming the vials were immediately immersed in a gallon of water at 25°C, whereas the slow warming vials were permitted to come to 25°C in room air.

TABLE 5

Shock Treatment of Microorganisms

Run	Sample	Shock Speed (meters/msec)	Temperature (°C)	Exposure Time (msec)	Numbers of Colonies Counted*			
					Organism	TGY	Sabourauds	Anaerobic
1	Mixed Cultures 99, 100, 233, 152 215, 232, 178	1.46	1050	2.1	152 232 99	~250 14 1		
2	Blank	1.45	1030	2.1	152 99	1 1		
3	Mixed Cultures 99, 100, 233, 152 215, 232, 178	1.67	1360	1.8	152 232	~500 ~2500		
4	Blank	1.42	980		152	2		
5	Blank	no record			152	1	0	
6	Mixed Cultures 99, 100, 233, 152 215, 232, 178	1.49	1090	2.1	152 232 233 100 178 99	~5000 ~40 0 0 0 0	0 0 1 4 2 1	
7	Blank	1.56	1200	2.0	152	4	0	0

TABLE 5 (cont.)

Run	Sample	Shock Speed (meters/msec)	Temperature (°C)	Exposure Time (msec)	Numbers of Colonies Counted*			
					Organism	TGY	Sabourauds	Anaerobic
8	Mixed Cultures 99, 100, 233, 152 215, 232, 178	1.57	1210	2.0	152 232 100	~150 ~80 0	0 0 2	~60 0 0
9	Blank	1.56	1190	2.0	152	5	0	3
10	Mixed Cultures 99, 100, 233, 152 215, 232, 178	no record			152 178 232 100	~600 8 ~50 0	0 4 0 6	~100 0 0 0
11	Blank	1.57	1210	2.0	152	3	0	0
12	Mixed Cultures 99, 100, 233, 152 215, 232, 178	1.56	1200	2.0	232 152 178 100	~800 ~400 2 0	0 0 4 8	0 ~100 0 0
13	Blank	1.56	1197	1.97	152	2	0	1
14	Pure Culture 152 Storage Time Study	1.56	1170	1.99	152 (1 min)~2000 152 (10 min)~2000 152 (100 min)~2000 152 (1000 min)~2000			

TABLE 5 (cont.)

Run	Sample	Shock Speed (meters/msec)	Temperature (°C)	Exposure Time (msec)	Numbers of Colonies Counted*			
					Organism	TGY	Sabourauds	Anaerobic
15	Blank	1.56	1200	1.98	152	11		
16	Blank	1.56	1200	1.98	152	11	0	0
17	Pure Culture 232	no record			152	6		
18	Blank	1.58	1218	1.95	152 Mold	2 1	0 0	0 0
19	Mixed Cultures 215, 233, 99	2.42	2865	1.24	215 233 99	0 0 ~50	0 16 0	10 0 0
20	Blank	1.58	1226	1.95	152	1	0	0
21	Pure Culture 99	1.56	1197	1.97	152 99	6 0	0 ~100	0 0
22	Blank	1.56	1197	1.97	99 Mold	2 0	0 2	0 0

TABLE 5 (cont.)

Run	Sample	Shock Speed (meters/msec)	Temperature (°C)	Exposure Time (msec)	Numbers of Colonies Counted*		
					Organism	TGY	Sabourauds	Anaerobic
23	Pure Culture 232 Storage Time Study	1.56	1187	1.98	232 (1 min) 0 232 (10 min) 0 232 (100 min) 0 232 (1000 min) 0			
24	Blank	1.56	1192	1.98		0	0	0
25	Pure Culture 232 Storage Time Study	1.56	1197	1.98	232 (1 min) 0 232 (10 min) 0 232 (100 min) 0 232 (1000 min) 0			

*When no values are given, the sample was not plated.

daily. In no case did more than 16 hours elapse between observations. These observations of the culture plates took place over a period of at least ten days; however, some plates were overgrown before ten days.

Bacillus globigii was recovered following all the runs where the sample was a mixed-culture sample. The number of colonies counted ranged from 150 to an estimated 5000. This culture was also recovered in all the blank runs except one. The number of colonies recovered from the blank runs, however, ranged from 1 to 11. Run 14 time study showed that the recovery of B. globigii after shock exposure was the same after storage of the sample for 1, 10, 100, and 1000 minutes at room temperature.

Serratia marcescens was also consistently recovered from test runs when mixed-culture samples were used. The number of recovered colonies of S. marcescens ranged from 14 to 2500. However, when this organism was shock tested in pure culture in runs 23 and 25, the organism was not recovered. Also, Serratia marcescens was never recovered following the blank runs.

Aspergillus niger was recovered in four out of the six mixed-culture runs and the number of observed colonies ranged from 2 to 8. A. niger was not recovered from any of the blank runs.

Streptomyces griseus was recovered in three out of the six runs with mixed-culture samples. The observed colonies ranged from 2 to 8 in number. S. griseus was not recovered from any of the blank runs.

Cladosporium resinae was recovered in only two out of the six runs where mixed-culture samples were used. Only one colony was observed in each case of recovery. One colony of culture was recovered from each of two blank runs which followed the mixed-culture runs.

Penicillium notatum was recovered only once from the six runs which used mixed-culture samples. Only one colony was observed in this one recovery.

Anaerobic Clostridium pasteurianum was tested for in three out of the six runs which used mixed-culture samples. It was not recovered from any of these three runs, nor was it recovered from any of the blank runs in which it was tested.

At the end of the mixed-culture runs, good data on the survival of Cladosporium resinae, Penicillium notatum, and Clostridium pasteurianum was not evident. The lack of conclusive evidence for survival of these organisms was believed to be due to overgrowth by the other organisms in the mixed sample. In all three of the runs using mixed-culture samples for which C. pasteurianum was tested, 60 to 100 colonies of the facultative aerobe Bacillus globigii were recovered. Since these organisms are morphologically very similar, microscopic examination did not conclusively demonstrate the presence of C. pasteurianum. Penicillium notatum and Cladosporium resinae were believed to be overgrown by other faster-growing organisms. Since more conclusive evidence of the survival of these three organisms was desired, a mixture of these three species was used as the sample in run 19.

The results of run 19 showed that these three organisms survived the shock treatment. Ten colonies of C. pasteurianum were observed. Since the colonies of this organism coalesced into a solid mass of growth in less than 48 hours, it is possible that more colonies appeared but

were not observed. Similarly, the 16 observed colonies of Penicillium notatum overgrew the plate thus masking the presence of other colonies. Approximately 50 colonies of Cladosporium resinae were recovered from this run.

However, before the Cladosporium resinae colonies were evident, run 21 using a pure culture of this organism was performed. It was suspected that the faster-growing Penicillium notatum might overgrow the Cladosporium resinae since Penicillium notatum appeared first on the Sabouraud agar plate of run 19. The result of run 21 showed the recovery of approximately 100 colonies of Cladosporium resinae.

4. DISCUSSION OF RESULTS

4.1 Determination of Survival

All seven species of microorganisms tested as a mixed-culture sample in the shock experiments were demonstrated to survive the shock wave treatment. Identification of the microorganisms was based upon both colony and morphological characteristics. In some cases the recovered microorganisms were transferred to other media to facilitate identification.

Although numbers of colonies were recorded it was not intended to quantitate survival. The purpose of recording numbers of colonies was to show relative numbers, especially when comparing the results of blank runs with sample test runs. In most cases where a small number of colonies were observed, the number was probably accurate. However, on occasion a faster-growing fungus could have overgrown slower-growing organisms. For example, where ten colonies of Clostridium pasteurianum were observed in a matter of hours, these colonies coalesced and obliterated the appearance of more colonies. In addition, where the estimate of the number of colonies was a large number, 50 or more colonies, a meaningful observation would have to have been made at the time that discrete colonies were formed and before the colonies coalesced. This difference in time was normally only a few hours.

Bacillus globigii and Serratia marcescens were chosen for the storage time study because they apparently survived the shock treatment more consistently and in greater numbers than the other organisms of the mixed-culture sample. B. globigii survived room storage of 1, 10, 100,

and 1000 minutes following the shock treatment and prior to placing the organisms on growth mediums. No difference was observed in the numbers of colonies which subsequently grew after different storage times.

Pure Serratia marcescens, on the other hand, did not survive room storage of even one minute. When mixed-culture samples were tested for survival of the shock treatment, at least one minute at room temperature elapsed before the filter paper could be placed on growth media. Since Serratia marcescens survived all these shock treatments when in the presence of the other test organisms, but did not survive as a pure-culture sample, it must be concluded that the presence of the other organisms in some way protected S. marcescens from the deleterious effects of the shock treatment. A portion of the pure-culture sample of S. marcescens was tested for viability at the time of introduction to the shock treatment, thus eliminating any question about sample viability. About 10^5 viable organisms were found by dilution pour plate from about one half of the sample introduced into the shock tube.

The data presented herein shows that all the microorganisms survived the shock treatment. However, before the results of cryogenic air sampling in the upper atmosphere can be interpreted, it is necessary to quantitatively determine the percent survival of microorganisms under such conditions. Determination of the percent survival before and after shock is necessary because it is impossible to collect the total sample by the presently-designed system. Quantitative information becomes critical to the success of this method of collection, because the expected level of microorganisms of the upper stratosphere is small and because the amount of air that can be sampled is limited.

The quantitative data can be obtained by one of several methods. To determine the percent survival of microorganisms introduced into the shock tube is relatively easy. A comparison of numbers formed by a standard plate and microscopic count of a sample of microorganisms to be introduced would give this value. However, to determine percent survival after the shock treatment is not as easy. Some possible methods for determining the percent survival after the shock treatment are:

(a) Developing an accurate method of eluting the microorganisms which have been impacted on the collecting filter.

(b) Impacting the microorganisms into a small amount of frozen air on the collecting surface, warming the system, and collecting the microorganisms in an impinger containing some suspending medium.

(c) Impacting the microorganisms on a solid surface such as stainless steel and then washing off the surface.

(d) Using radio-labelled microorganisms.

4.2 Shock Wave Calculations

The temperatures and contact times in the shock tube were calculated from the ideal shock equations reported in Section 2.3.2. For the case of powdered solids introduced into the gas stream, the gas velocity will be slightly larger than the true particulate velocity because of the inertia of the powdered sample. The contact time of the powdered sample will therefore be slightly larger than that calculated from these equations. This effect will be small for long-particle travel times[4] and the contact times reported here are therefore based on the gas velocities calculated from the given equations.

Introduction of powdered samples into the shock heated gas will also result in a slight reduction of the gas temperature by absorption of heat by the sample. This effect is very difficult to access since it will be a function of the amount, constitution, and particle size of the sample. The effect of particulate on the temperature of the shocked gas in reflected shock waves has been measured and shown to decrease by less than 10% from those calculated from the incident shock velocity measurements.[5] Therefore, the temperatures reported here are also those calculated for the case of air with no particulate present.

Both of the effects discussed above, i.e. the increase of the contact time and the lowering of the gas temperature with the introduction of particulate into the shocked gas, will occur upon passage of particulate through the shock front of a supersonic vehicle and the magnitude of the effects should be similar to those encountered here. The approximations made here are therefore doubly justified, firstly because they are small effects and secondly because they will be similar to those occurring during actual collection in the atmosphere.

4.3 Determination of Immersion Times

In order to determine the viability of the microorganisms after freezing to 20°K it was deemed necessary to determine the immersion times required to reduce the temperature of the vials containing the lyophilized organisms to the desired temperature. It was estimated that dunking the vials into the liquid hydrogen would cause the evolution of larger quantities of hydrogen gas than could be safely handled in the closed facility. Studies of immersion times in liquid nitrogen were therefore

conducted in order to obtain an estimate of this time in liquid hydrogen. The experimentally determined curve for the rate of cooling in liquid nitrogen is shown in Figure 7. The rate-of-cooling curve for liquid hydrogen was determined by shifting the exponential curve to coincide with the boiling point of liquid hydrogen. The immersion times used in the studies were appreciably longer than the times estimated from this curve in order to be absolutely certain that the vials had reached the desired temperature of 20°K.

5. CONCLUSION

The results obtained in this study definitely established that the majority of the selected species of microorganisms survived the conditions of the shock wave and cryogenic collection. The only exception was the doubtful survival of Serratia marcescens in a pure-culture sample. Thus, it is indicated that the collection of microorganism could be successfully accomplished under the following conditions:

(a) near vacuum of the upper atmosphere

(b) rapid heating by the shock wave of a rocket vehicle

(c) high-speed impaction on a solid surface

(d) rapid cooling to 20°K

(e) rewarming to room temperature and subsequent storage for a period of time

However, further studies are necessary before quantitative data can be obtained.

6. REFERENCES

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